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The Nuclear Matrix Protein, NRP/B, Acts as a Transcriptional Repressor of E2F-mediated Transcriptional Activity

ORIGINAL ARTICLE

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Background: NRP/B, a family member of the BTB/Kelch repeat proteins, is implicated in neuronal and cancer development, as well as the regulation of oxidative stress responses in breast and brain cancer. Our previous studies indicate that the NRP/B-BTB/POZ domain is involved in the dimerization of NRP/B and in a complex formation with the tumor suppressor, retinoblastoma protein. Although much evidence supports the potential role of NRP/B as a tumor suppressor, the molecular mechanisms of NRP/B action on E2F transcription factors have not been elucidated.
Methods: Three-dimensional modeling of NRP/B was used to generate point mutations in the BTB/Kelch domains. Tet-on inducible NRP/B expression was established. The NRP/B deficient breast cancer cell line, MDA-MB-231, was generated using lentiviral shNRP/B to evaluate the effect of NRP/B on cell proliferation, invasion and migration. Immunoprecipitation was performed to verify the interaction of NRP/B with E2F and histone deacetylase (HDAC-1), and the expression level of NRP/B protein was analyzed by Western blot analysis.Changes in cell cycle were determined by flow cytometry. Transcriptional activities of E2F transcription factors were measured by chloramphenicol acetyltransferase (CAT) activity.
Results: Ectopic overexpression of NRP/B demonstrated that the NRP/B-BTB/POZ domain plays a critical role in E2F-mediated transcriptional activity. Point mutations within the BTB/POZ domain restored E2-promoter activity inhibited by NRP/B. Loss of NRP/B enhanced the proliferation and migration of breast cancer cells. Endogenous NRP/B interacted with E2F and HDAC1. Treatement with an HDAC inhibitor, trichostatin A (TSA), abolished the NRP/B-mediated suppression of E2-promoter activity. Gain or loss of NRP/B in HeLa cells confirmed the transcriptional repressive capability of NRP/B on the E2F target genes, Cyclin E and HsORC (*Homo sapiens* Origin Recognition Complex).
Conclusions: The present study shows that NRP/B acts as a transcriptional repressor by interacting with the co-repressors, HDAC1, providing new insight into the molecular mechanisms of NRP/B on tumor suppression.
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Key Words: NRP/B, E2F transcription factor, Transcriptional activity, Breast cancer

INTRODUCTION

Nuclear organization represents the dynamic networks and three-dimensional architectures of chromosomes and their regulatory components. The nuclear matrix and its associated proteins mediate the process of chromosome organization through the folding and looping of chromatin. Recent studies have demonstrated the role of nuclear matrix proteins in RNA processing, stress responses, cell adhesion, survival and transcriptional regulation as either activators or repressors.[1-8] The nuclear matrix provides the foundation on which transcription factors operate.[9] Indeed, transcription factors, including tumor suppressors such as p53 and pRb are dynamically associated with specific nuclear matrix sites which support their functional complex formations.[10-14] Although aberrant expression or genetic mutation of nuclear matrix proteins have been found in many

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cancers,[15-19] very little is known regarding the molecular mechanism by which nuclear matrix proteins are involved in tumorigenesis.

We have previously characterized a nuclear matrix protein, NRP/B, also termed ENC1,[20,21] which is highly expressed in insoluble nuclear fractions and contains two major structural elements: a BTB/POZ domain and 115-amino acid motif in the predicted N-terminus and "Kelch" repeats consisting of about 50 amino acids in the predicted C-terminal domain. NRP/B has been implicated in neuronal differentiation,[20,22] and malignant transformation, including glioblastoma, astrocytoma,[16,23,24] and colorectal cancer.[25,26] Mutations in NRP/B contribute to brain and colorectal tumorigenesis by promoting cell proliferation and decreasing cellular apoptosis.[16,25] In addition, NRP/B has been found to be involved in oxidative stress responses, both in human breast cancer and primary brain tumors.[3,4] The combination of sequence homology analysis and a three-dimensional model indicates several potential protein-protein interaction sites for NRP/B, via the BTB/POZ domain. Targeted mutagenesis studies have demonstrated that the N-terminus of the BTB/POZ domain of NRP/B is important for its dimerization.[22]

The BTB/POZ domain is found primarily in zinc finger-containing transcription factors and mediates both homo- and hetero-dimerization in vitro.[22,27-29] The BTB/POZ family is implicated in transcriptional repression, tumorigenesis, cell proliferation, and maintenance of embryonic stem cell pluripotency.[30-33] A new mammalian BTB/POZ domain protein, DP-interacting protein, interacts with the DP component of the E2F heterodimer and is physically located as speckles in the nuclear envelope region.[34] In *Drosophila melanogaster*, transcription factors containing the BTB/POZ domain play a key role in a variety of developmental events such as photoreceptor development, muscle recognition by nerve cells and limb development.[35] A new human BTB/POZ domain protein, Fanconi Anemia Zinc Finger (FAZF), is homologous to the promyelocyte zinc finger protein (PLZF), which represses the transcription of specific targets by recruiting histone deacetylase (HDAC) through the SMART-mSin3-HDAC co-repressor complex. Both FAZF and PLZF are localized in nuclear speckles. FAZF is also a transcriptional repressor that is able to bind to the same DNA target sequence as PLZF[36] and forms a heterodimer with PLZF.

The transcription factor E2F, a key element in the control of cell proliferation, regulates several families of genes whose products are required for cell cycle progression, such as cyclin E,[37] or for DNA synthesis,[38] such as dihydrofolate reductase.[39] E2F, in turn, plays an essential role in the G1-S transition.[40] pRb and other pocket binding proteins suppress E2F-mediated transcription in cells when they progress through the early G1 phase.[41,42] Furthermore, the pRb-E2F repressive complex functions in association with HDAC.[43-48] HDACs essentially repress transcription, probably through the deacetylation of histone tails.[49] Ferreira et al. (2001) reported that HDAC-1 is stably bound to an E2F target promoter during the early G1 phase in proliferating cells and is released at the G1-S transition, where pRb is known to be a transcriptional co-repressor, playing a key role in regulating cell proliferation and differentiation.[50]

In this study, we examined the role of NRP/B as a growth suppressor through the inhibition of human papillomaviral protein E2 promotor activity.[51] Immunoprecipitation analysis indicated that the association of NRP/B with E2F and HDAC-1 were cell cycle dependant. Furthermore, NRP/B suppression of E2 promoter activity was mediated through the BTB/POZ domain but not through "Kelch" repeats of NRP/B. The suppressive activity of NRP/B on E2F transcription was relieved by amino acid mutations (F45A, T46A, D47A, H60A, R61D, L64A) within the BTB/POZ domain. Moreover, treatment with the HDAC inhibitor, trichostatin A (TSA) restored the E2 promoter activity initially inhibited by NRP/B. Thus, NRP/B-mediated growth suppression appears to be directly related to the inhibition of E2F-mediated transcription through the recruitment of a corepressor complex, including HDAC-1.

MATERIALS AND METHODS

1. Materials

The generation of monoclonal antibodies, VD2, against NRP/B was described.[20] The mouse ascites fluid containing monoclonal antibodies was further purified on a protein G-Sepharose column. The purified antibodies were quantified using extinction coefficient 1.46 for 1 mg/mL antibody solution. Monoclonal anti-ENC1/NRP/B and pRb antibodies were purchased from BD Bioscience (San Jose, CA, USA). Antibodies, E2F1 and HDAC1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Cell culture

PC-12, Neuro2A, HEK 293, HeLa, and MDA-MB-231 cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). HEK 293, HeLa, Neuro2A, MDA-MB-231 cells were grown in DMEM medium, supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and penicillin/streptomycin (Invitrogen). PC-12 cells were grown in RPMI medium supplemented with 10% horse serum and 5% FBS. We

previously generated PC-12 Tet-on/off cell lines stably expressing NRP/B.[22] The Tet-on/off PC12 clones were maintained as described.

3. Three-dimensional modeling of NRP/B

Both BLAST and AlignMaster programs used the PLZF BTB/POZ domain as a three-dimensional template. The structure of the human PLZF BTB/POZ domain was obtained from the Protein Data Bank (Protein Data Bank code 1BUO and 1CS3A). Superimposition, model building, construction of insertion regions, structure validation and calculation of structural properties was carried out using subprograms ProMod v3.5, SPDBV v3.5, Loop v2.60, LoopDB v2.60, Parameters v3.5 and Topologies v3.5 which are available in the Automated SwissModel Package Program (www.expasy.ch/swissmod). The three- dimensional model of the NRP/B Kelch domain was generated from residue ranges between amino acids 292 through 575 of the conserved region in the Kelch domain, based on template 1zgkA by the use of BLAST2 and Cn3D program (www.ncbi.nlm.nih.gov, National Institute of Health).

4. Construction of NRP/B expression vectors

NRP/B-GFP constructs were generated by standard methods. Briefly, full-length NRP/B cDNA was subcloned into the BamHI and HindIII sites of the pEGFP-N2 vector (Clontech, Palo Alto, CA, USA) by polymerase chain reaction (PCR) to generate 3'-end GFP ligated to NRP/B cDNA. The pNRP/B-delC2-GFP construct encoding amino acids 296-589 was fused with GFP and was generated by PCR using NRP/B-specific oligonucleotides containing a restriction site linker and then subcloned into the BamHI and HindIII sites. pE2-chloramphenicol acetyltransferase (CAT) constructs were obtained from Dr. Mary R. Loeken (Josulin Diebetic Center, Boston, MA, USA). pCAG NRP/B was constructed by insertion of full-length NRP/B into EcoR1 and Not1 sites of pCAG obtained from Dr. Suzanne Topalian (National Cancer Institute, Bethesda, MD, USA). Generation of lentiviruses was performed as previously described.[52] Lentiviral shNRP/B (ENC1) expression vectors were purchased from Sigma (St. Louis, MO, USA). Cyclin E and HsOrC)-luciferase constructs were purchased from AddGene (Cambridge, MA, USA).

5. Immunoprecipitation and western blot analysis

Immunoprecipitation and Western blot analysis were performed as previously described.[20]

6. Flow cytometry analysis

Cells were transiently transfected with NRP/B-GFP wild-type (wt) using lipofectamine 2000 (Invitrogen) as described in the manufacturer's manual. Cells were harvested 48 h after transfection. Cell cycle analysis was performed as previously described.[20]

7. Chloramphenicol acetyltransferase assay and luciferase reporter assay

CAT assays were performed as previously described.[53] Nuclear extracts were assayed in a volume of 150 μL. All enzyme activities were assayed within the linear range. Percent conversion was determined by liquid scintillation quantitation of ^{14}C counts/min in acetylated and nonacetylated chloramphenicol spots from the chromatography plate. For the luciferase reporter assay, HeLa cells were split at 5×10^4 cells into 12-well plates and co-transfected with luciferase reporter constructs and target genes, such as E2F or NRP/B, alone with 5 ng of RL-CMV using Effectene Transfection reagent according to the manufacturer's instructions (Qiagen, Gaithersburg, MD, USA). Whenever one of the DNA components was eliminated, the total amounts of DNA were balanced with a pcDNA3 empty vector. All transfections were performed in triplicates. After 24 hours, cells were lysed in passive lysis buffer and 20 μl were used to measure the activity of E2 and *Renilla* luciferase with dual luciferase reporter assay system (Promega, Milwaukee, WI, USA).

8. Quantitative RT-PCR

Total RNA was purified using Trizol Reagent (Invitrogen) and 1 μg of total RNA was reverse transcribed in a 50 μl reaction using TaqMan Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA, USA). 2 μl of the reverse transcribed cDNA was subjected to PCR according to Applied Biosystems technical recommendations. TaqMan probes for CyclinE and HsOrC were purchased from assay-on-Demand (Applied Biosystems). PCR primers for NRP/B were forward 5'-CAGTGGTGGCCTGAAAGAGA-3' and reverse 5'-TGCAGACAGTAAGGCTGGCACT-3'. Triplicate reactions were run for each RNA sample.

9. Wound healing migration assay

Wound healing migration assay was performed using a 35 mm μ-Dish (ibidi GmbH, Munich, Germany). 70 μl of DMEM containing 35,000 cells was seeded into each chamber of the cell culture insert. After 24 hours, the cell culture insert was gently removed with sterile tweezers, and 1 ml of fresh medium was added to the 35 mm μ-Dish. The cell migration toward the gap region was observed under an inverted light microscope (Olympus IX51, Olympus America Inc., Melville, NY, USA), and

the images of each μ-Dish were taken after 0, 3, 5, 9, and 24 hours.

10. MTT assay

Cell viability was assessed by MTT assay. MDA-MB-231 cells were seeded at a concentration of 1×10^4 cells/ml in 96-well plates. The cells were incubated for 48 hours in medium supplemented with 10% FBS before the experiments. MTT solution (5 mg/ml) was added to each well after 24, 48, and 72 hours and incubated at 37°C to allow the formation of formazan crystal. After 2 hours, the media was carefully aspirated from the wells, and 200 μl of DMSO (dimethyl sulfoxide) was added to dissolve the crystals. The absorbance at 570 nm was measured by spectrophotometry. All MTT assays were performed in quadruplicate.

11. Chamber cell-migration assay

8.0 μm pore size PET (polyethylene terephthalate) track-etched membrane with Falcon cell culture insert (Becton Dickinson, Bedford, MA, USA) was used for chamber cell migration assay. A total number of 5×10^4 cells (500 ul) were added to the upper chamber, and 700 ul of fresh medium was added to the lower chamber. The chambers were incubated for 24 hours at 37°C, and the remaining cells on the upper surface

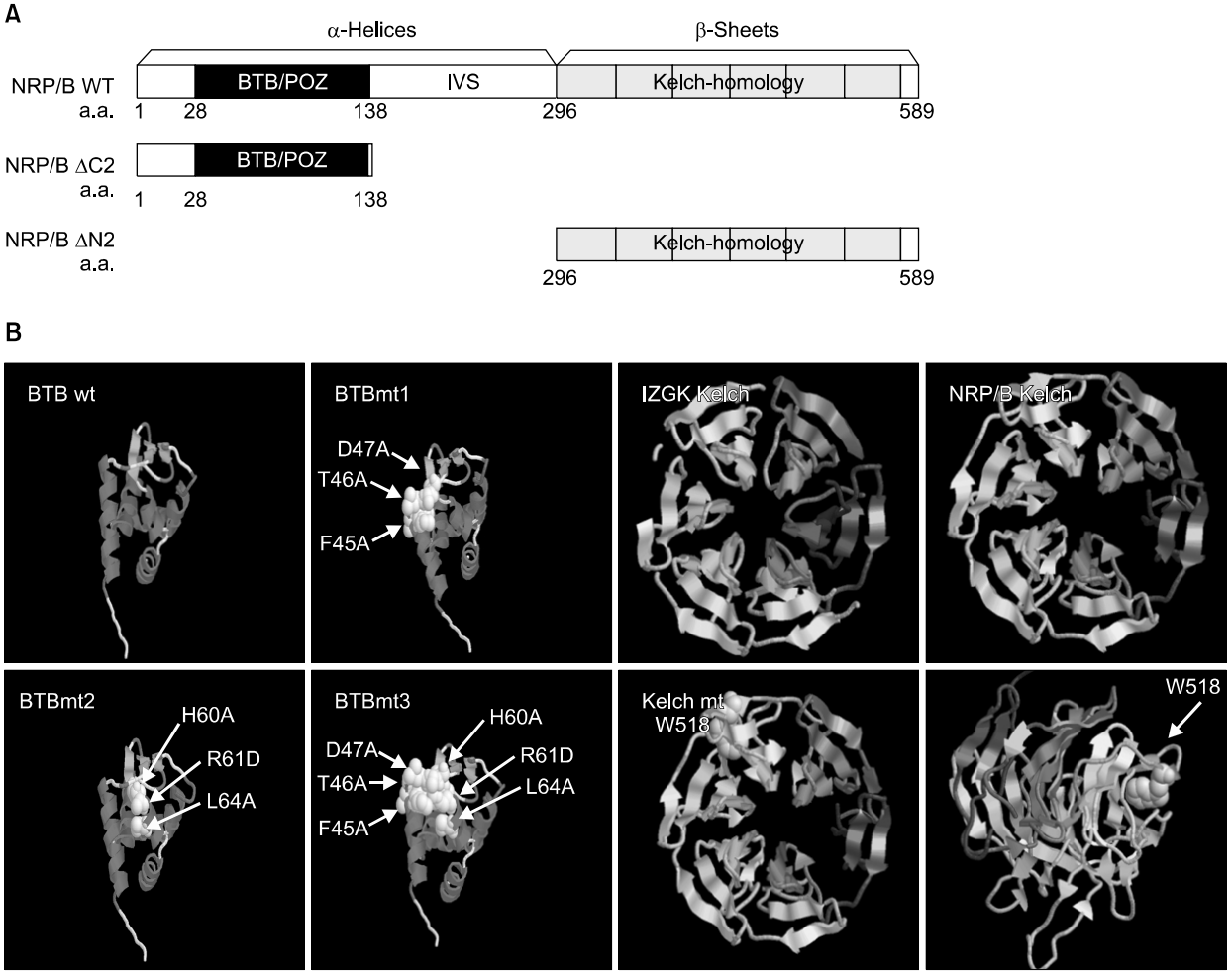

Figure 1. Generation of NRP/B BTB and Kelch mutants based on three-dimensional modeling. (A) Schematic presentation of NRP/B protein and deletion mutants. IVS, intervening sequence. Numbers indicate amino acid residues. (B) Three-dimensional modeling and mutation in the BTB and Kelch domains. BTB mutants are as follows; mutant 1(mt1): Asp (D) 47 to Ala (A), Thr (T) 46 to Ala (A) and Phe (F) 45 to Ala (A); BTB mt2: His (H) 60 to Ala (A), Arg (R) 61 to Asp (D) and Leu (L) 64 to Ala (A); BTB mt3: mutations in mt1+ mt2. Red colors indicate a-helices and yellow colors indicate b-sheet structures. Mutation sites are shown as white balls. The Kelch domain of IZAGK was used as a template. The NRP/B Kelch mutant includes Try (W) 518 to Ala (A). Thick arrows with green, yellow, blue, red, sky blue and orange colors indicate b-sheet of the Kelch repeat domain. Mutation sites are shown as sky-blue spheres. ⊿C2, C-terminal deletion mutant; ⊿N2, N-terminal deletion mutant.

of the membrane were removed with cotton swabs and gently washed with 500 ul of PBS. Cells that migrated through the membrane and attached to the lower surface of the membrane were fixed and stained with 70% ethanol and 1% toluidine blue solution. Stained cells were counted under microscopic observation to measure the average number of cells that had migrated.

RESULTS

1. Three-dimensional modeling of NRP/B and the generation of point mutations in NRP/B domains

NRP/B contains both an α-helical BTB/POZ domain and a β-sheet Kelch repeats structure (Fig. 1A). We designed several mutants based on previously generated three-dimensional models of the NRP/B BTB/POZ domain (amino acid residues 13-146)[22] and six Kelch repeat domains (amino acid residues 294-533). For the modeling of the Kelch domain, we used a crystal

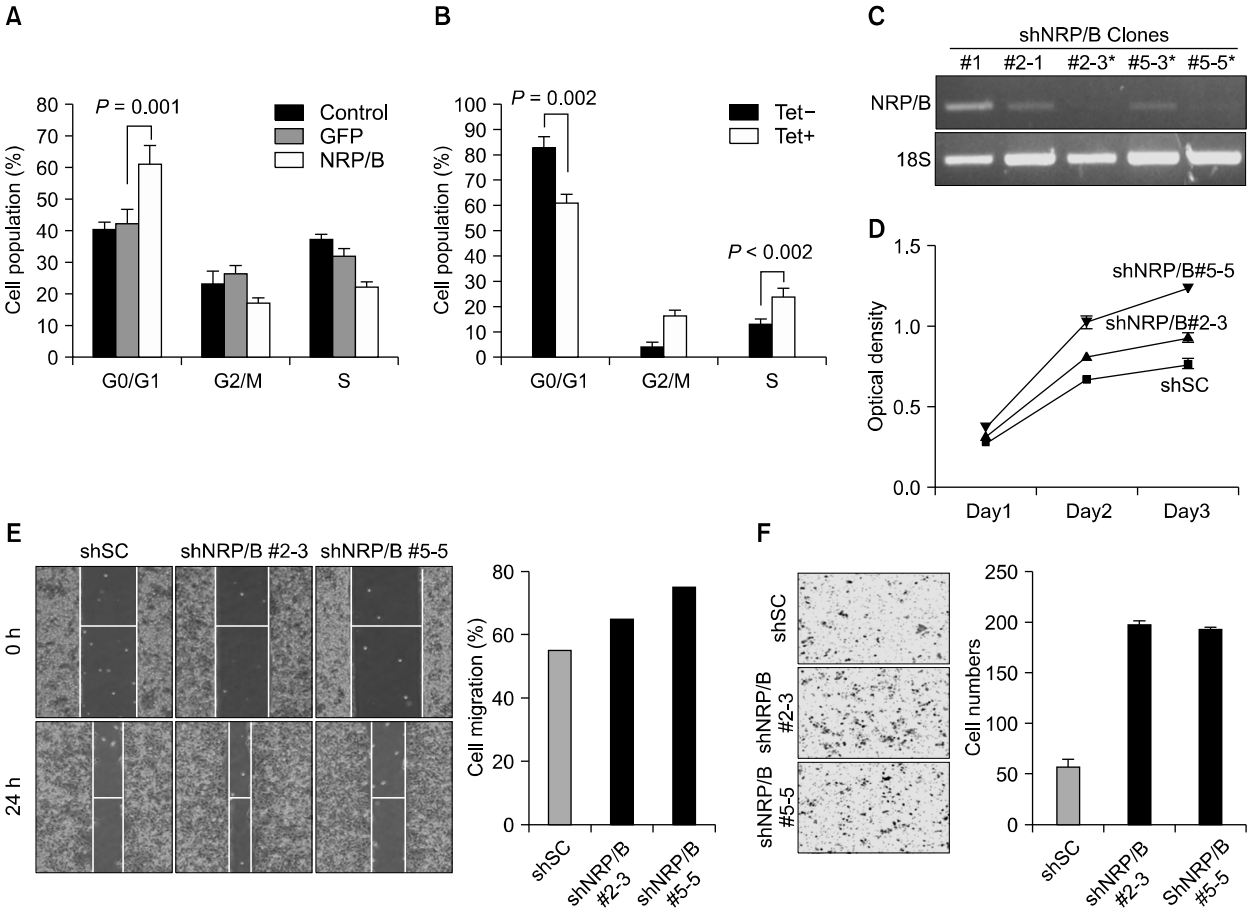

Figure 2. NRP/B regulated cell proliferation and migration. (A) Mouse neuroblastoma Neuro2A cells transfected with vectors expressing pEGFP and NRP/B-GFP, were sorted out separately from the non-transfected population. Control cells were non-trasnfected cells. The parameters are averages of triplicate samples. (B) NRP/B Tet-off PC12 clones (SC10, SC11, SC13) were grown in the presence or absence of tetracycline for 24 hours supplemented with 10% Tet system approved FBS (Clontech, Palo Alto, CA, USA). Cells were then analyzed by Flow cytomery as indicated in "Experimental procedures". The results are mean values of triplicate samples from NRP/B PC12 clones. P-values were obtained by paired t-tests as shown. (C) Expression of NRP/B in MDA-MB-231 cells infected with lentiviral shNRP/B. (D) Loss of NRP/B enhanced cell proliferation. Cell viability was measured in shNRP/B clones of MDA MB-231 cell by MTT assay after 24, 48, and 72 hours. shSC stands for shScramble Control. (E) Cell migration toward the gap area was photographed at 0 and 24 hours. Perpendicular lines indicate the borders between gaps, and horizontal lines represent the width of gaps. The percentage of cell migration was calculated as follows: (W-w)/W x 100%: Width of gap (W at T = 0 hour) and width (w at T = 24 hours). The quantitative results of wound healing migration assay are presented on the right panel. (F) Representative images of the chamber cell migration assay show the effect of NRP/B on cell migration after toluidine blue staining of migrated cells. For quantification, cells were counted in three randomly determined fields. The data on the graph are the average number of migrated cells in the indicated cell lines.

structure of ZGK Kelch as a template.[54] Loops were generated between Pro376-Ala380, Leu389-Leu393 and Ala401-Ser411 within NRP/B Kelch repeats domain. The final total energy was −2339.067 KJ/mol. The calculated high energy level indicated that the Kelch domain needs to be stabilized by binding partners such as actin and suggests a possible critical site for actin binding in the Kelch domain of NRP/B. The final three-dimensional models of NRP/B and its mutants are shown in Figure 1B. The sites for point mutations are as follows; BTB mutant (mt) 1 (F45A, T46A, D47A), BTB mt2 (H60A, R61D, L64A), BTB mt3 (F45A, T46A, D47A, H60A, R61D, L64A) and Kelch mt1 (W518A). All the mutants contained at least one conserved residue of either BTB/POZ or "Kelch" repeats. The three-dimensional analysis of the NRP/B BTB/POZ domain indicated a potential pocket-formed structure that can be used as protein-protein interaction sites. Therefore, mutations in the BTB domain may change the ability of NRP/B to interact with other proteins via this domain. Moreover, this pocket site contains highly conserved amino acids, indicative of its evolutionary conserved function.

The "Kelch" model shows critical sites for actin binding. W518 is located outside of the superbarrel structure to maintain the three-dimensional structure of the Kelch domain. W518 is also a highly conserved amino acid in the Kelch superfamily, and its charges, location and solvent accessibility strongly suggests its significance in the actin binding properties of the Kelch domain.

2. The NRP/B played an important role in the regulation of cell proliferation and migration

Our previous studies demonstrated that NRP/B is involved in neuronal differentiation and proliferation.[20,22] However, the molecular mechanisms of NRP/B involvement in these cellular functions have not been elucidated yet. To examine the effect of NRP/B on cell proliferation, GFP-tagged full-length NRP/B wt was transfected into mouse neuroblastoma Neuro2A cells (Fig. 2A). GFP positive cells were sorted and analyzed for cell cycle profiles. FACS analysis showed that cells transfected with NRP/B wt were increased by 18-22% in the G0/G1 phase cell population (P = 0.001) and decreased by 10-15% in the S phase cell population in comparison to the control and GFP-transfected cells. We further examined the growth suppressive effect of NRP/B using Tet-on/off PC12 clones. Induction of NRP/B in the absence (Tet-off) or presence (Tet-on, data not shown) of tetracycline or doxycycline significantly (P = 0.002) enhanced the G1/G0 population by 15-20%, depending on the Tet-off PC12 clones used (Fig. 2B). Similar data was obtained from Tet-on PC12 clones (data not shown). Furthermore, to examine the loss of NRP/B function on cell proliferation and migration, shNRP/B MDA MB-231 cell lines were generated using lentiviral shNRP/B expression vector. Expression of NRP/B was confirmed by RT-PCR (Fig. 2C). The cell viability rates of the shNRP/B clone #2 and #5 were gradually increased depending on the incubation period for up to three days compared to the control shSC cells (Fig. 2D). Wound healing migration assay and chamber cell-migration assay proves that the loss of NRP/B amplified the migratory properties of MDA-MB-231 cell lines, suggesting that the NRP/B deficiency in MDA-MB-231 breast cancer cell line promotes advanced cell proliferation and tumorigenesis (Fig. 2E and 2F).

3. Endogenous interaction of NRP/B with E2F transcription factors and HDAC-1

The BTB domain of NRP/B specifically interacts with the TR subdomain within the B pocket of pRb and overexpresssion of NRP/B induces hyper-phospholylation of pRb.[22] Tumor suppressive effects of pRb are known to mediate the inhibition of E2F transcriptional activity via its complex formation with E2F and HDACs during cell cycle progression.[41-44,55] To analyze if the cell cycle inhibitory effect of NRP/B is mediated through pRb-HADC transcriptional repressor complexes, we examined the endogenous interaction of NRP/B with transcription factor E2F and the transcriptional suppressor HDAC-1 in both quiescent and proliferating cells. Growth arrested HeLa cells via serum deprivation was stimulated with 10% serum for 12 hours and 24 hours where cells move from G1 to S phase respectively (Fig. 3A). Immunoprecipitation of NRP/B showed that endogenous NRP/B interacted with E2F and HDAC1 at late G1/S after 12 hours of serum stimulation. As cells entered S phase, HDAC1 was dissociated from NRP/B-E2F complex (Fig. 3B). Co-transfection of NRP/B with other HDACs demonstrated that NRP/B also interacted with HDAC2 and 5 (data not shown). Hence, the interaction between NRP/B with E2F and/or HDAC-1 provides new partners for NRP/B in transcriptional regulation.

4. NRP/B inhibited E2F-mediated transcriptional activity

To examine the effect of NRP/B on E2F-mediated gene transcription, E2 promoter/enhancers were used (Fig. 3C). E2-CAT wt and deletion mutant constructs were co-transfected with plasmids expressing NRP/B into HeLa cells. NRP/B suppressed wt E2 and E2-80/70 promoter activity by 20-25%, and E2-80/70/−45/36 promoter activities by -30%. There was no significant change in E2-64/60, −45/36 (Fig. 3D and 3E). The effect of NRP/B on these E2 mutant CAT activities indicated that NRP/B

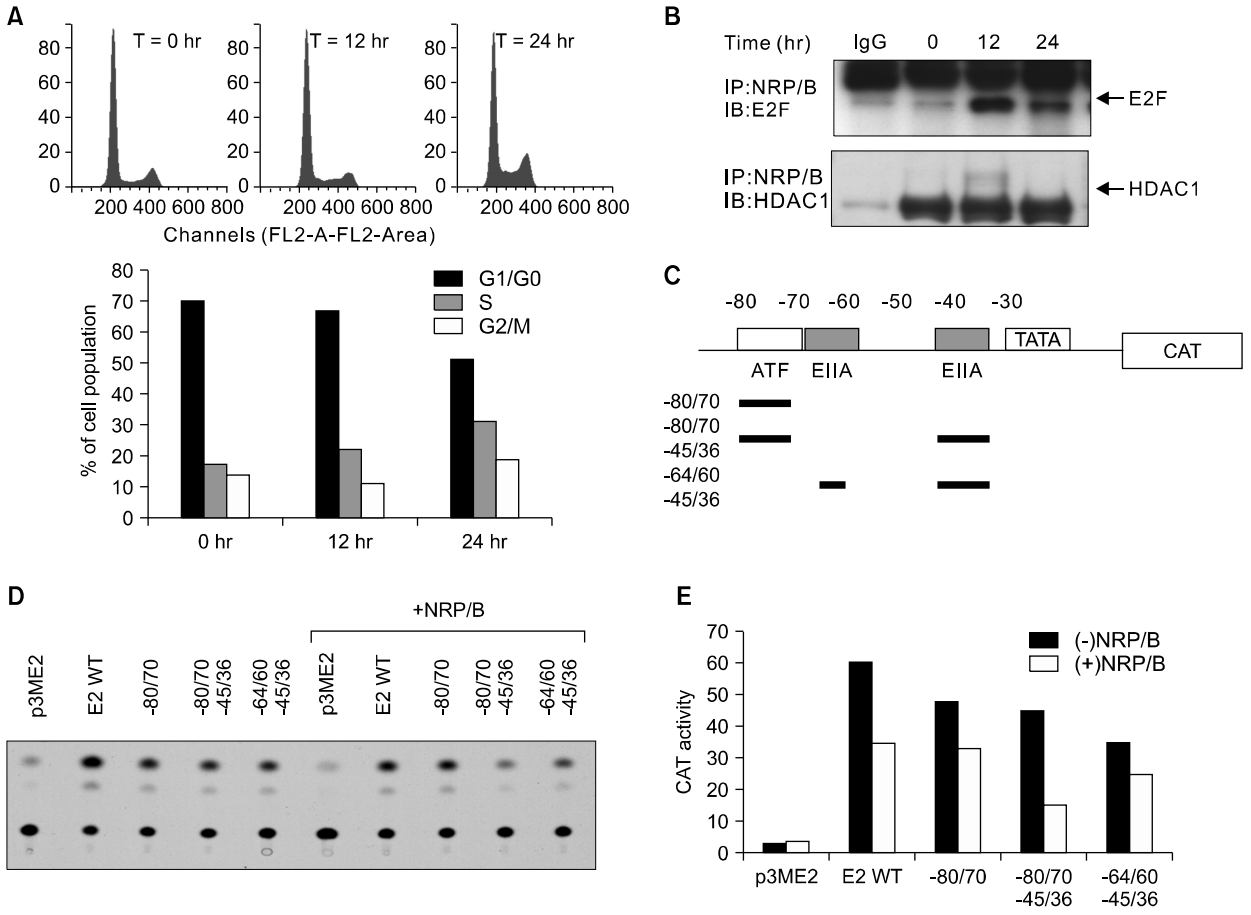

Figure 3. NRP/B inhibits E2-promoter activity. (A) Immunoassociation of NRP/B with transcription factor E2F and with HDAC-1. Growth-arrested HeLa cells via serum deprivation (T = 0 hour) were stimulated with 10% fetal bovine serum for 12 and 24 hours. Total cell extracts (500 μg) were subjected to immunoprecipitation with monoclonal anti-NRP/B antibody and mouse IgG. Blot was probed with anti-E2F antibody and reprobed with anti-HDAC1 antibody. (B) Flow cytometry analysis. Cells in the same condition as above (A) were subjected to cell cycle analysis as described in "Experimental procedures". Graph in the bottom panel indicates the quantification of the cell cycle profile. (C) Schematic diagram of the E2 promoter/enhancer. The position of the ATF binding site (from −82 to -66) is indicated by an empty box over the wild-type (wt) sequence. (D) Inhibition of specific E2 promoter activity by NRP/B in CAT assay. HeLa cells were co-transfected with E2-CAT wt or E2-CAT mutant constructs in the presence or absence of NRP/B, together with a β-galactosidase expression vector. p3M-E2, which contains EIIA promoter sequences (−30 to +40), was used as a control. Extracts were prepared 48 hours after transfection and assayed for CAT activity as well as β-galactosidase activity. (E) CAT activity is expressed as a percentage conversion of acetylated chloramphenicol, normalized to β-galactosidase activity in the above samples. IB, Immunoblot; IP, Immunoprecipitation; p3ME2, control vector; TATA, TATA box.

specifically inhibited E2 promoter sites, but does not affect ATF sites (Fig. 3C-3E). To identify the specific inhibitory site, previously described NRP/B deletion and BTB point mutation constructs were co-transfected with the E2-CAT construct. E2 promoter activity was inhibited by 65-78% with NRP/B wt and delC2 mutant (Fig. 4A and 4B). However, NRP/B delN2 consisting of the Kelch motif did not show any significant effect on E2 promoter activity. Co-transfection of BTB mt 1 (F45A, T46A, D47A), mt 2 (H60A, R61D, L64A) or mt 3 (F45A, T46A, D47A, H60A, R61D, L64A) with the E2-wt promoter construct showed that mutations in $_{45}$FTD$_{47}$ and $_{60}$HR$_{61}$D$_{64}$ (BTB mt 3) dramatically abolished the suppression of E2F transcriptional activity, while mt 1 and 2 showed partial inhibition (40-50%) (Fig. 4A and 4B). Therefore, E2 promoter activity is mediated through the BTB domain of NRP/B, specifically within the regions of amino acids, $_{45}$FTD$_{47}$ and $_{60}$HR$_{61}$L$_{64}$.

Accumulating evidence indicates that pRb represses E2F transcriptional activity by recruiting a HDAC.[43-48] HDACs act as transcriptional repressors by binding to E2F target promoters during the early G1 phase in proliferating cells.[50] Therefore, we investigated whether the transcriptional suppression of NRP/B is mediated through the recruitment of an HDAC repressor. To examine this, a specific inhibitor of HDAC-1, TSA, was used. E2F transcriptional activity inhibited by NRP/B (1 μg) was restored in

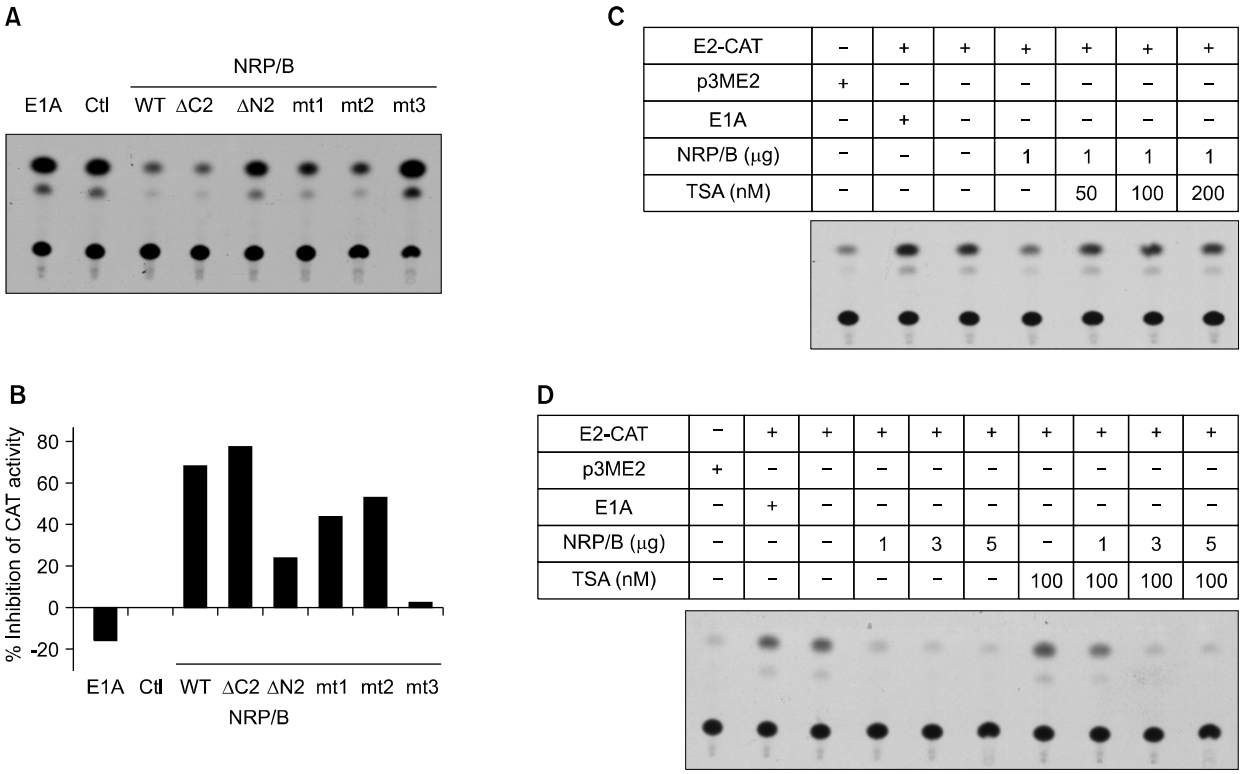

Figure 4. Mutation in BTB domain and trichostatin-A (TSA) treatment restores E2-promoter activity suppressed by NRP/B. (A) E2 promoter activity was measured with extracts obtained from the transient transfection of NRP/B deletion mutants (C2 or N2) and expression vectors containing point mutation in the NRP/B-BTB domain (NRP/B mt1, mt2 and mt3). NRP/B C2 contains the BTB/POZ domain whereas N2 contains only "Kelch" repeats. Control (Ctl) indicates the transfection of pE2-CAT wild-type alone. Cotransfection of E1A was used as a positive control. (B) Percent inhibition of E2-CAT activity was calculated by comparing CAT activities to the activity of the control (depicted as 0%), normalized to β-galactosidase activity from the same samples of experiment. (C) Effect of TSA on NRP/B-mediated transcriptional repression of E2F transcriptional activity. Cells were transfected with E2-CAT and/or NRP/B (1 μg). Transfected cells were then treated with TSA (50, 100 and 200 nM) for 24 hours. Extracts were prepared 48 hours after transfection. E2-CAT and β-galactosidase activities were measured as described in "Experimental procedures". (D) E2 promoter activity was measured following the transfection of pE2-CAT wt, E1A, or NRP/B expression vector (1, 3, and 5 μg), and a β-galactosidase expression vector in the presence or absence of TSA (100 nM). Transfected cells were treated with TSA for 24 hours and extracts were subjected to CAT assay. Ctl, Control; ⊿C2, C-terminal deletion mutant; ⊿N2, N-terminal deletion mutant; p3ME2, E2 control palsmid.

cells treated with TSA (50 nM), with an 80-95% increase in E2 promoter activity (Fig. 4C and 4D). The inhibitory effect of NRP/B on E2 promoter activity showed concentration dependant properties. With an increase of NRP/B concentration, the E2 promoter activity was significantly inhibited by 70-90% as compared to E2-CAT alone (Fig. 4D). TSA (100 nM) alone increased E2F promoter activity by 40% and recovered over 90% of the E2 promoter activity inhibited by NRP/B. However, in the presence of high concentration of NRP/B (3-5 μg), TSA treatment did not inhibit NRP/B-mediated suppression of E2 promoter activity (Fig. 4D). Taken together, these results indicate that NRP/B served as a transcriptional repressor in E2 promoter activity by recruiting HDAC-1.

5. Regulation of E2F-target gene promoter activity and gene transcription via NRP/B

To determine whether the transcriptional suppressive activity of NRP/B correlates to regulation of E2F target genes, we first examined the effect of NRP/B on the promoters of Cyclin E and HsOrC. As shown previously in Figure 3 and 4, NRP/B inhibited E2F-mediated transcriptional activity. E2F enhanced the activity of two E2F target gene promoters, HsOrC and Cyclin E by 2 to 6 folds, respectively (Fig. 5A and 5B). In addition, ectopic overexpression of NRP/B in HeLa cells significantly inhibited basal and E2F-mediated Cyclin E and HsOrC transcriptional activities (Fig. 5A and 5B). Furthermore, we used the lentiviral-based NRP/B and shNRP/B to examine the gain and loss of NRP/B function on E2F target gene expression in HeLa cells. Expression of NRP/B was

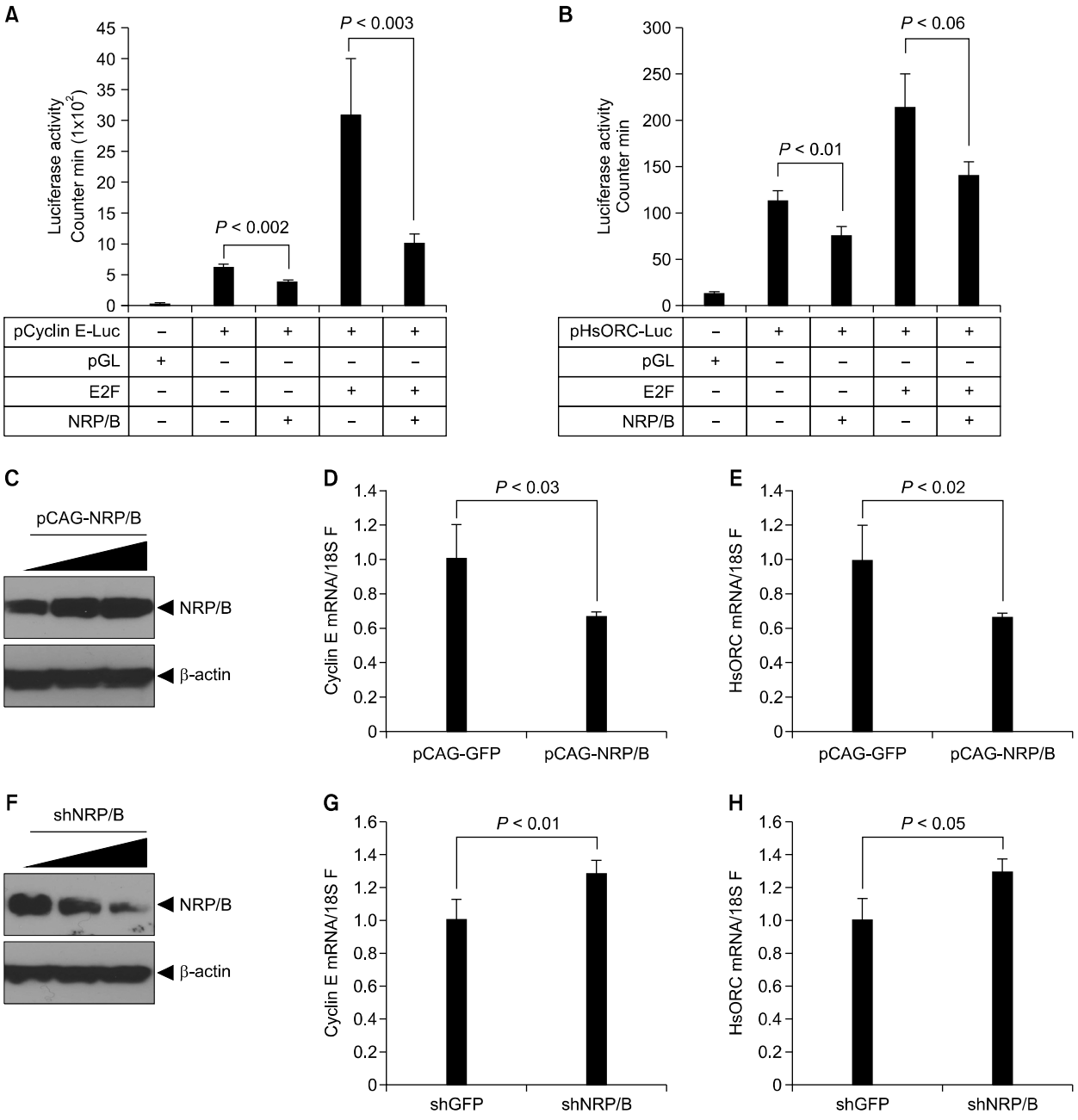

Figure 5. NRP/B inhibits E2F-target gene promoters and gene transcription in HeLa cells. (A and B) Luciferase activity in HeLa cells after transfection with Cyclin E (A) and HsOrC. (B) reporter constructs, along with E2F or NRP/B as indicated. pGL, an empty reporter vector was used as a control. The experiments were repeated with triplicate samples at least three times and the representative experiment is shown. (C and F) Expression of NRP/B protein in HeLa cells infected with increasing amount of lentiviral-pCAG-NRP/B (C) and lentiviral-shNRP/B (F). 30 μg of total cell lysates were subjected to western blot analysis. (D, E, G and H) qRT-PCR of Cyclin E (D and G) and HsOrC (E and H). Total mRNA was prepared from cells infected with lentiviral-pCAG-NRP/B and lentiviral-shNRP/B as shown in (C and F). Viral products from pCAG-GFP (D and E) and shGFP (G and H) were used as controls. The results are mean values of relative mRNA levels normalized to 18S ± SEM from triplicate samples. P-values were obtained by paired t-tests as shown. Luc, luciferase.

gradually increased by lentiviral-NRP/B infection and markedly decreased by shNRP/B expression (Fig. 5C and 5F). qRT-PCR analysis showed that the ectopic expression of NRP/B significantly suppressed cyclin E ($P < 0.03$) and HsOrC ($P < 0.02$) gene transcript (Fig. 5D and 5E). Loss of NRP/B by shNRP/B showed a significant increase of Cyclin E ($P < 0.01$) and HsOrC ($P < 0.05$) gene transcripts (Fig. 5G and 5H). These results further validate the role of NRP/B as a transcriptional suppressor in E2F-mediated

transcriptional activity.

DISCUSSION

NRP/B is known to exert its effect on neuronal differentiation and proliferation through its association with the key molecules in cell cycle, such as pRb.[20] We have shown previously that the NRP/B-BTB/POZ domain mediated the association of NRP/B with pRb through the TR subdomain within the B pocket of the pRb protein.[22] Mutations in the BTB domain significantly reduced the NRP/B-pRb interaction and dimerization of NRP/B. The present study indicates that the NRP/B had significant growth inhibitory properties (Fig. 2A and 2B). After transfection of NRP/B-GFP into Neuro2A and HEK 293 cells, the population of G0/G1 was increased by 15-20% in comparison to non-transfected and mock (GFP only) transfected cells. The growth regulatory effect of NRP/B was also verified in several Tet-on/off PC12 clones (Fig. 2B). We found that the induction of NRP/B in Tet-on/off cell lines significantly delayed the kinetics of cell cycle progression in comparison to control cells (unpublished data). Interestingly, long-term (two to four days) ectopic overexpression of NRP/B in Tet-on/off PC12 clones induced the neuronal differentiation of PC12 in which pRb became hyper-phosphorylated.[22] On the other hand, the loss of NRP/B in MDA-MB-231 cells showed a marked increase in cell proliferation and migration, which validates the growth inhibitory function of NRP/B, suggesting that NRP/B may have tumor suppressive properties. Regulation of E2F-mediated transcription may lead to a better understanding of the molecular mechanisms of cellular progression. The activity of the E2F transcription factor in cells is tightly regulated, partly through complex formations with a number of key regulators of cellular proliferation and cell cycle control. Interaction of pRb with E2F in the G0/G1 phase is known to inhibit cell proliferation.[20,22,23] Since NRP/B interacts with E2F, HDAC1 and pRb depending on cell cycle progression, it is crucial to understand the molecular mechanism of NRP/B action on these growth regulatory proteins. Our studies showed that NRP/B suppressed E2 promoter activity, which was mediated by the BTB domain but not by the Kelch motif. Within the NRP/B-BTB domain, amino acids ($_{45}$FTD$_{47}$, and $_{60}$HR$_{61}$L$_{64}$) were important in regulating E2 promoter/E2F binding activities. Among these amino acids, $_{46}$TD$_{47}$ are known to be conserved and essential for the transcriptional suppression of PLZF. Furthermore, the inhibition of HDAC1 activity via treatment of TSA restored E2F transcriptional activity initially suppressed by NRP/B, suggesting that the complex formation of NRP/B-E2F-HDAC1 in the late G1/S phase (Fig. 3A and 3B) may lead to transcriptional suppression. It is conceivable that the recruitment of HDAC1 and/or the hypophosphorylated form of pRb[20] could engage the E2F-transcriptinal machinery. Indeed, NRP/B seems to be involved in E2F transcriptional suppression, by interacting with HDAC1-E2F. Earlier studies have shown that the cell cycle-dependant recruitment of HDAC-1 is correlated with the deacetylation of histone H4 on a pRb-E2F target promoter.[50] The histone methylase SUV39H1 and the methylase-lysine binding protein HP1 are involved in the repressive functions of pRb.[56] Thus, the pRb functions, at least partly, as a repressor through the recruitment of HDAC activity.[40,46,47] Regulation of the E2F-mediated transcriptional activity via NRP/B was further examined in both E2F target gene promoters (Cyclin E and HsOrC) and gene transcription, indicating that ectopic overexpression of NRP/B significantly inhibited not only the promoter activities but also the gene transcription of the E2F target genes, Cyclin E and HsOrC (Fig. 5). However, loss of NRP/B by infection of lentiviral shNRP/B enhanced E2F target gene transcription respectively. NRP/B could possibly impair the formation of E2F complex in order to interrupt the activation of E2F-mediated transcription. NRP/B could indirectly bind to E2F sites through the formation of a DNA-protein complex that contains E2F1, pRb and HDACs.

Taken together, NRP/B acts as a transcriptional suppressor. Inhibition of E2F-mediated transcription through NRP/B interaction with co-repressors may significantly impact cell proliferation and tumorigenesis.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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